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## Modulation of Src Activity by Low Molecular Weight Protein Tyrosine Phosphatase During Osteoblast Differentiation

Willian F. Zambuzzi<sup>1</sup>, José M. Granjeiro<sup>2</sup>, Kaushal Parikh<sup>3</sup>, Saravanan Yuvaraj<sup>3</sup>, Maikel P. Peppelenbosch<sup>3</sup> and Carmen V. Ferreira<sup>1</sup>

<sup>1</sup>Laboratory of Bioassays and Signal Transduction, Department of Biochemistry, Institute of Biology, University of Campinas (UNICAMP), Campinas, <sup>2</sup>Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense (UFF), Niterói, RJ, <sup>3</sup>Department of Cell Biology, Groningen University Medical Center, University of Groningen, Groningen

### Key Words

Covalent modulation • Low molecular weight protein tyrosine phosphatase • Osteoblast differentiation • Src kinase

### Abstract

**Background:** Src kinase plays a critical role in bone metabolism, particularly in osteoclasts. However, the ability of Src kinase to modulate the activity of other bone cells is less well understood. In this work, we examined the expression and activity of Src and low molecular weight protein tyrosine phosphatase (LMWPTP) during osteoblast differentiation and assessed the modulation of Src kinase by LMWPTP. **Methods:** Differentiation of MC3T3-E1 pre-osteoblasts was induced by incubation with ascorbic acid and  $\beta$ -glycerophosphate for up to 28 days. Src phosphorylation and LMWPTP expression were analyzed by immunoblotting. Src dephosphorylation in vitro was assessed by incubating immunoprecipitated Src with LMWPTP followed by assay of the residual Src activity using Sam68 as substrate. The importance of LMWPTP in Src dephosphorylation was confirmed by silencing pre-osteoblasts with siRNA-LMWPTP and then assessing Src phosphorylation.

**Results:** Pre-osteoblast differentiation was accompanied by a decrease in phosphorylation of the activator site of Src and an increase in phosphorylation of the inhibitory site. The expression of total Src was unaltered, indicating that post-translational modifications play a pivotal role in Src function. LMWPTP expression was higher in periods when the activator site of Src was dephosphorylated. LMWPTP dephosphorylated pY<sup>527</sup>-Src and pY<sup>416</sup>-Src in vitro, with greater specificity for pY<sup>527</sup>-Src. Activation of LMWPTP produced strong activation of Src mediated by fast dephosphorylation of pY<sup>527</sup>-Src, followed by slower deactivation of this kinase via dephosphorylation of pY<sup>416</sup>-Src. **Conclusion:** These results provide new insight into the mechanisms governing the dynamics of Src activity during osteoblast differentiation. A fuller understanding of these mechanisms will improve our knowledge of bone metabolism and of the regulation of Src in other types of cells.

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### Introduction

Cells respond to internal and external stimuli through integrated networks of signaling pathways that involve cascades of sequential phosphorylation or dephosphor-

ylation mediated by the action of protein kinases and protein phosphatases. Specifically, tyrosine phosphorylation is generally accepted as a critical regulator of a variety of cell biological processes, including cell proliferation, migration, differentiation, and survival [1].

Of the various protein tyrosine kinases currently known, Src kinase has an important role in physiological and pathological processes such as cell survival, differentiation, tumorigenesis and inflammation [2]. Src activity is also important in bone homeostasis since targeted disruption of the c-Src gene in mice causes osteopetrosis by accelerating osteoblast differentiation, whereas osteoclasts failed to resorb bone because they did not form ruffled borders [3-5].

The activity of Src-family kinases (including Src, Lyn, Fyn, Yes, Lck, Blk and Hck) is tightly regulated by their phosphorylation status [6, 7]. Protein tyrosine kinases are generally maintained in an inactive conformation by the phosphorylation of Y<sub>527</sub>Src, six residues from the c-terminus of c-Src. Under basal conditions *in vivo*, 90-95% of Src is phosphorylated at Y<sub>527</sub>Src [8, 9]. Following dephosphorylation of Y<sub>527</sub>Src, Src undergoes intermolecular autophosphorylation at Y<sub>416</sub>, a residue present in the activation loop; the phosphorylation of this residue promotes kinase activity and association of the kinase with substrate molecules [10]. Some PTPs (CD45, RPTP $\alpha$ , PTP1B, Shp1/2, PTP $\epsilon$ , PTP $\kappa$  and LMWPTP) can influence Src family tyrosine kinase activity by dephosphorylating Y<sub>527</sub>Src [11]. In recent years, LMWPTP has been found to tightly control the activity of Src and *vice versa* [12, 13]. Thus, overexpression of LMWPTP dramatically decreased Src kinase activity in response to stimulation by PDGF [12]. LMWPTP occurs in the same complex as Src, as shown by co-immunoprecipitation experiments, and acts as a substrate for Src. The phosphorylation of Y<sub>131</sub> in LMWPTP by Src increases the enzymatic activity of this phosphatase by more than 25-fold [14-18]. In contrast to the activation of Src, well-defined candidates for the deactivation of this kinase have not yet been identified. Indeed, the mechanisms governing the transient nature of Src kinase signalling remain poorly understood.

Based on these considerations, in this work we examined the modulation of Src by LMWPTP during the differentiation of pre-osteoblast cells. We also investigated the modulation of the activity of these enzymes in *in vitro* assays. RNA silencing and phosphatase/kinase activity assays revealed a dichotomic regulation of Src tyrosine kinase activity by LMWPTP. This is the first

report to show that LMWPTP is involved in Src activation during osteoblast differentiation. Additional studies are required to establish the importance of this regulation in different pathologies.

## Materials and Methods

### Reagents

Antibodies against total-Src (#2108), phosphoY<sub>416</sub>Src (#2101), phosphoY<sub>527</sub>Src (#2105), non-phospho-Y<sub>416</sub>Src (#2102), non-phospho-Y<sub>527</sub>Src (#2107), pan-actin (#4968) and anti-rabbit, anti-goat and anti-mouse peroxidase-conjugated antibodies were purchased from Cell Signaling Technology (Beverly, MA). Sam68 (Src substrate) and antibody against phospho-tyrosine ( $\alpha$ -pY, #sc-508) were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA). Antibody against LMWPTP was from Abcam. The antibodies were used at dilutions recommended by the manufacturers. Recombinant human LMWPTP (rhLMWPTP) was developed by one of the authors (JMG, unpublished data). All chemicals were of analytical grade.

### Cell culture

MC3T3-E1 pre-osteoblasts (subclone 4) obtained from the American Type Culture Collection (ATCC) were routinely grown in modified alpha minimum essential medium ( $\alpha$ -MEM; Sigma Chemical Co., St. Louis, MO) without ascorbic acid, supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and 1% penicillin-streptomycin, in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. The cells were plated at an initial density of 50,000 cells/cm<sup>2</sup> and after 24 h (day 0) differentiation was induced by adding osteogenic medium (OM) containing 50  $\mu$ g of ascorbic acid/ml and 10 mM  $\beta$ -glycerophosphate for 28 days. The osteogenic medium was replaced every three days.

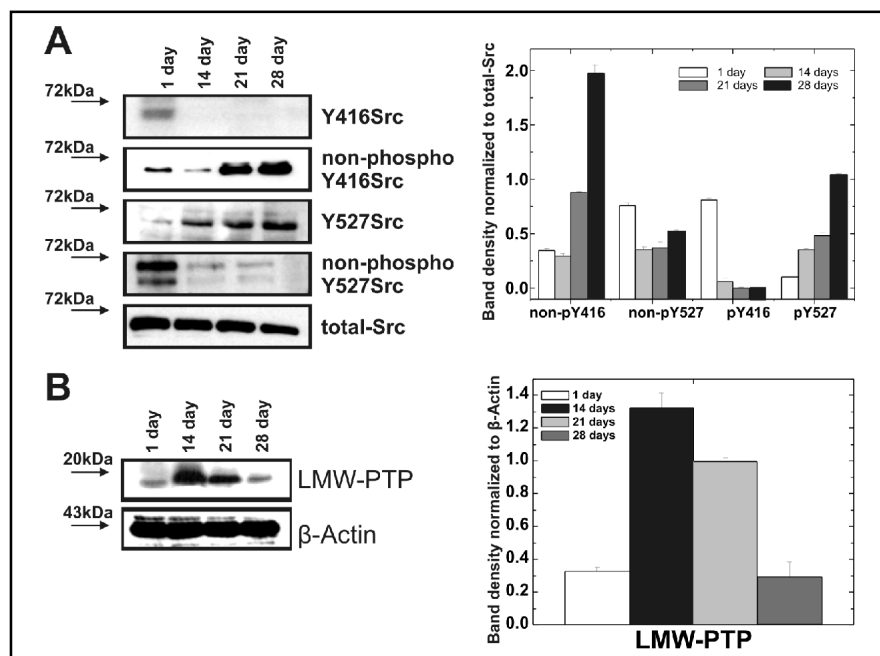
### Cell fractionation

Cytoskeletal fractions and soluble cellular fractions were separated by using RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 10  $\mu$ g of aprotinin/ml, 10  $\mu$ g of leupeptin/ml). Complete RIPA lysis buffer (cRIPA) consisted of RIPA plus 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate. Cellular fractionation was done as described by Cirri *et al.* [19].

### Transfection of MC3T3-E1 cells with LMWPTP

MC3T3-E1 cells grown to 60% confluence (6-well plates) were transiently transfected with LMW-PTP. The transfections were done using an Effectene transfection kit (QIAGEN Benelux, Venlo, Netherlands) according to the manufacturer's instructions. Briefly, the cells were transfected with 2.4  $\mu$ g of pcDNA3.1/V5-His-TOPO vector with or without an insert containing the sequence of human LMWPTP after a cytomegalovirus promoter. Overexpression was always verified by western blotting.

**Fig. 1.** Phosphorylation of Src and LMWPTP expression during osteoblast differentiation. Src phosphorylation (A) and LMWPTP expression (B) were evaluated by western blotting. Soluble lysates were matched for protein content and analyzed by western blotting. The western blots were probed with actin antibody to ensure equal protein loading.



#### Transfection of MC3T3-E1 cells with siLMWPTP

MC3T3-E1 cells grown to 60% confluence (6-well plates) were transiently transfected with siLMWPTP. The transfections were done using an Hiperfect transfection kit (QIAGEN Benelux, Venlo, Netherlands) according to the manufacturer's instructions. Briefly, the cells were transfected with LMWPTP siRNA (final concentration: 5 nM) or MAPK1 siRNA (positive control) for 72 h, and then scraped into 100 µl of cell lysis buffer (described in section 2.6), sonicated and spun down. The lysates were subsequently mixed with loading buffer for electrophoresis. The efficiency of transfection was assessed based on the expression of LMWPTP by western blot analysis (mean reduction in expression: 58.3%).

#### Immunoblotting and immunoprecipitation

Protein extracts were obtained by using a lysis cocktail (50 mM Tris-HCl, pH 7.4, 1% Tween 20, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM *O*-vanadate, 1 mM NaF and protease inhibitors - 1 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mM phenylmethanesulphonyl fluoride) for 2 h on ice. An equal volume of 2 x SDS gel loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue and 20% glycerol) was added to the samples and boiled for 5 min. Protein extracts were separated by SDS-PAGE in 12% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes.

For immunoprecipitation, the cells were lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 60 mM *n*-octylglucoside, 2 mM EDTA, 1 mM orthovanadate, 100 mM NaF, and proteases inhibitors). After centrifugation, the lysates were incubated overnight with anti-pan-Src or phospho-tyrosine antibody at 4°C and then incubated (rotatory mixing) with Protein A-Sepharose at 4°C for 2 h. The beads were washed three times with lysis buffer and twice with phosphate-buffered saline (PBS), after which

the immunoprecipitates were used for enzymatic assays and/or SDS-PAGE and immunoblotting as described above. Immunoreactive bands were detected with enhanced chemiluminescence (ECL) kits.

#### Phosphatase and kinase activities in vitro

**Phosphatase activity:** Cells were treated with freshly prepared pervanadate solution (10 µM) for 30 min and pan-Src was immunoprecipitated from the cellular lysate (1.5-3.5 mg of protein/ml). The immunoprecipitate (total Src) was subsequently incubated with rhLMWPTP at 37°C for 15 min or 30 min and the reaction then stopped by adding pervanadate.

**Kinase activity:** Kinase activity was assayed by adding 2 µg of Sam68 (protein substrate for Src) and 100 µM ATP to the samples from the dephosphorylation experiments (described above) followed by incubation at 37°C for 30 min. The reaction was terminated by adding loading buffer and the amount of Sam68 and its degree of phosphorylation were assessed by immunoblotting.

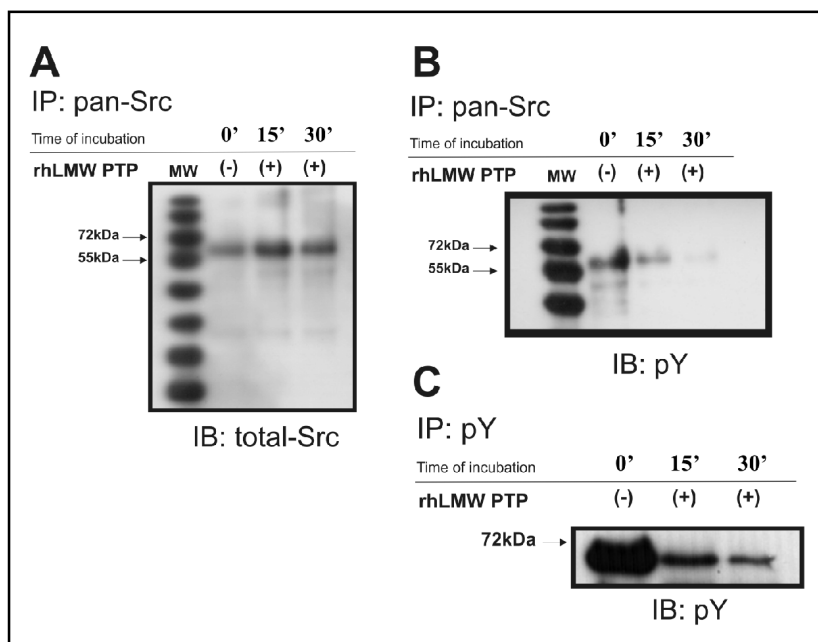
#### Cellular redox status

Cells were treated with H<sub>2</sub>O<sub>2</sub> (1 µM), L-NAME (3 µM) and GSH (5 mM) for 1 h and the cRIPA fraction was collected. Src phosphorylation (anti-pY<sub>416</sub>Src) and LMWPTP expression in the lysates were analyzed by western blotting. The extent of LMWPTP phosphorylation was assessed by immunoprecipitating the enzyme followed by western blotting with α-pY antibody.

## Results

Based on the critical role of Src kinase in bone homeostasis and its ability to interact with LMWPTP, in

**Fig. 2.** LMWPTP dephosphorylates Src at tyrosine residues. Cells were pretreated with pervanadate for 30 min (to increase pY-Src levels) and pan-Src (i.e. non-phosphorylated and phosphorylated-Src) was immunoprecipitated and incubated with rhLMWPTP *in vitro* for different lengths of time. (A) Equal amounts of pan-Src were used in the various experimental conditions, as assessed by western blotting with a pan-Src antibody (left panel). MW = molecular weight marker lane. (B) Western blots of immunoprecipitated rhLMWPTP-treated Src probed with pY antibodies. (C) Western blots of immunoprecipitated rhLMWPTP-treated Src probed with  $\alpha$ -pY antibodies.

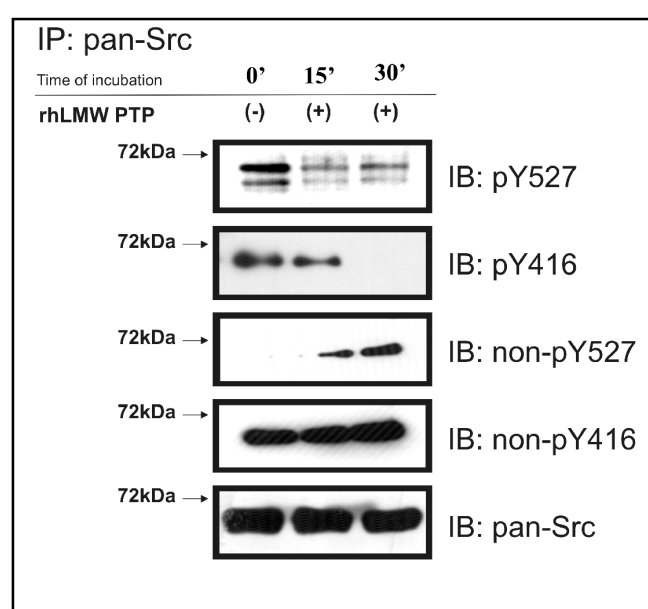


this work we examined the extent of Src phosphorylation status and LMWPTP expression during the differentiation of MC3T3-E1 pre-osteoblasts induced by ascorbic acid and  $\beta$ -glycerophosphate. We also assessed the dephosphorylation of Src by LMWPTP *in vitro*. The use of ascorbic acid and  $\beta$ -glycerophosphate to induce the differentiation of pre-osteoblasts (MC3T3-E1 cells) is well described in the literature and usually occurs after 21-28 days [20]. In this work, successful differentiation was confirmed by the increase in alkaline phosphatase activity and osteocalcin expression up to 28 days (data not shown).

#### *Covalent modulation of tyrosine phosphorylation determines Src kinase activity during osteoblast differentiation*

Src activity and its modulation by LMWPTP during osteoblast differentiation were examined in MC3T3-E1 pre-osteoblasts initially maintained under osteogenic conditions for 28 days. Subsequently, western blotting with specific antibodies showed that Src kinase was inactive during osteoblast differentiation since Y<sub>416</sub>-Src at the activator site was dephosphorylated (accompanied by a marked increase in non-phospho-Y<sub>416</sub>-Src) whereas Y<sub>527</sub>-Src at the inhibitory site was phosphorylated (accompanied by a marked decrease in non-phospho-Y<sub>527</sub>-Src) (Figure 1A). These findings indicated that Src kinase activity changed during osteoblast differentiation although there was no marked alteration in Src expression during this process.

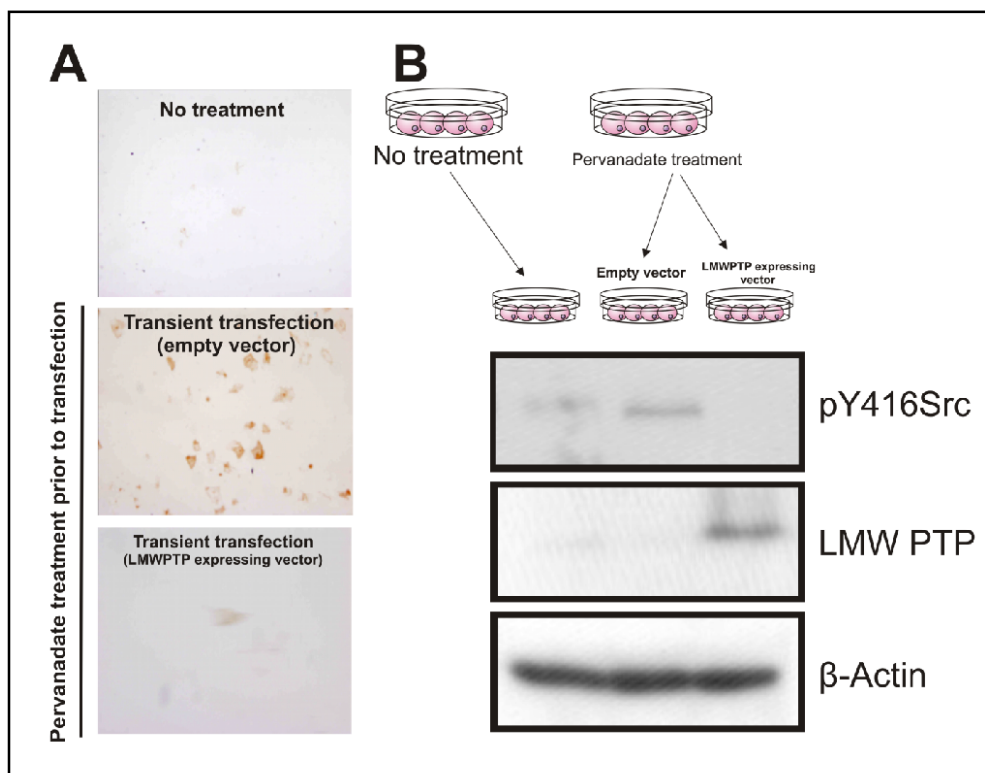
In contrast to Src, the expression of LMWPTP



**Fig. 3.** LMWPTP dephosphorylates pY<sub>416</sub>-Src and pY<sub>527</sub>-Src. To identify the tyrosine residues of Src that were dephosphorylated by LMWPTP, total Src was immunoprecipitated and incubated with rhLMWPTP for various times. Subsequently, pan-Src, phospho-Src (pY<sub>527</sub>-Src and pY<sub>416</sub>-Src), and non-phospho-Src (Y<sub>527</sub>-Src and Y<sub>416</sub>-Src) were analyzed by western blotting.

varied during osteoblast differentiation, with the highest expression being detected after 14 and 21 days (Figure 1B). Based on these findings, we speculated that LMWPTP could influence Src activity during bone formation.

**Fig. 4.** Decreased pY<sub>416</sub>-Src levels in MC3T3-E1 cells overexpressing LMWPTP. (A) Immunocytochemical analysis of pY<sub>416</sub>-Src levels. To increase pY<sub>416</sub>-Src levels, cells were incubated with pervanadate for 30 min before transfection with empty vector or an LMWPTP-expressing construct, after which the medium was replaced by one without pervanadate. (B) Western blot analysis of pY<sub>416</sub>-Src and LMWPTP levels following transient pervanadate treatment and overexpression of LMWPTP.



#### *Evidence for pY<sub>416</sub>-Src dephosphorylation via a tyrosine phosphatase-dependent mechanism*

The transient nature of Src activation in response to various stimuli during bone formation is still poorly understood. One possible explanation for the transient Src activity could be that pre-osteoblasts contain PTPs that dephosphorylate pY<sub>416</sub>-Src, thereby deactivating the kinase. Evidence for such activity in these cells was obtained from experiments in which MC3T3-E1 cells were pretreated with the pan-PTP inhibitor pervanadate (10  $\mu$ M) for 30 min. This treatment strongly increased pY levels in general, and that of pY<sub>416</sub>-Src in particular (not shown). The ability of pervanadate to block the dephosphorylation of pY<sub>527</sub>-Src indicated that MC3T3-E1 cells contain strong pY<sub>416</sub>-Src dephosphorylating activity. This conclusion was supported by the observation that these cells contained PTP activity towards pY<sub>416</sub>-Src and suggested that this phosphatase was a plausible negative regulator of c-Src activity.

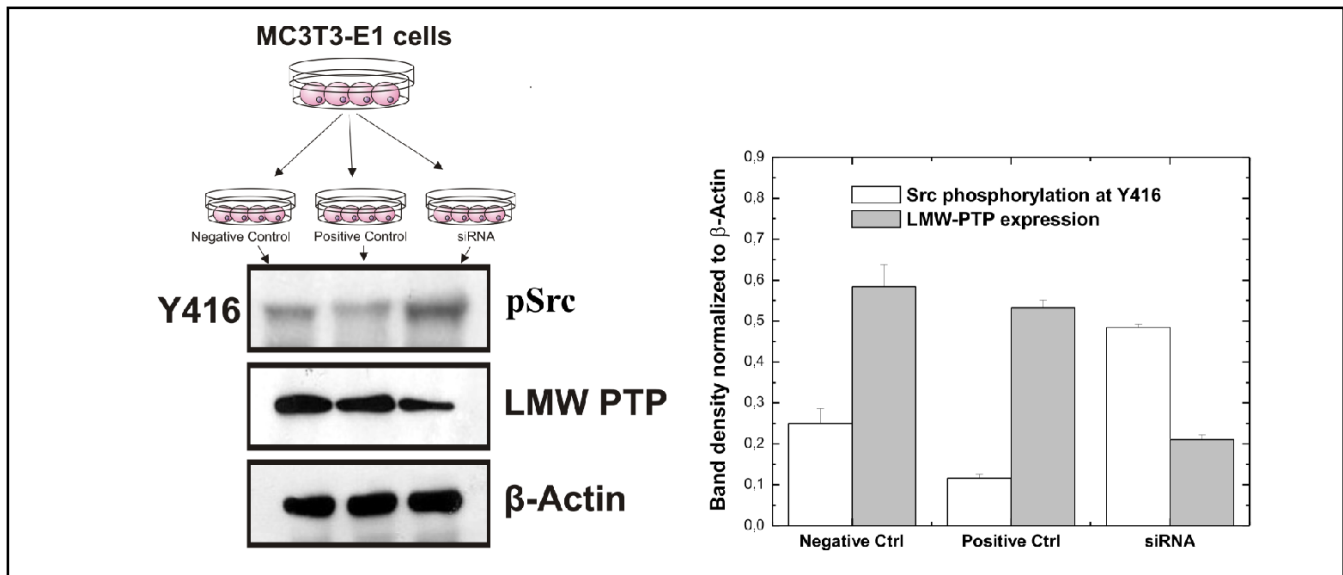
#### *pY<sub>416</sub>-Src is a substrate for LMWPTP*

Immunoblotting with anti-Src and anti-phosphotyrosine antibodies was used to investigate the action of rhLMWPTP on pY<sub>416</sub>-Src following the co-incubation of pSrc with rhLMWPTP for 15 min and 30 min. Pervanadate-treated MC3T3-E1 cells were used

as a source of pSrc. Figure 2A shows the levels of total Src during the reaction with rhLMWPTP. Immunoblotting with anti-phospho-tyrosine (pY) antibody revealed a decrease in phosphorylation after incubation with rhLMWPTP (Figure 2B), indicating that LMWPTP was able to dephosphorylate immunoprecipitated pSrc and that this enzyme may be a negative regulator of this kinase. This finding was supported by a decrease in the levels of pY in a protein band with a molecular mass similar to pSrc in immunoprecipitates incubated with rhLMWPTP (Figure 2C).

#### *LMWPTP dephosphorylates Y<sub>416</sub>-Src and Y<sub>527</sub>-Src in vitro*

Based on the finding that pSrc was a substrate for LMWPTP, we subsequently examined the preference of this PTP for pY<sub>416</sub>-Src and pY<sub>527</sub>-Src. By using a phosphatase assay similar as that described above, we found that both tyrosine residues of Src were substrates for LMWPTP (Figure 3). However, LMWPTP showed greater specificity for pY<sub>527</sub>-Src since there was a marked decrease in the level of this phosphorylated residue within 15 min when compared with pY<sub>416</sub>-Src. This decrease in phosphorylation was accompanied by a corresponding increase in non-phosphorylated Y<sub>527</sub>-Src.



**Fig. 5.** Effect of LMWPTP knock down with siRNA on pY<sub>416</sub>Src levels. MC3T3-E1 cells grown to 60% confluence were transiently transfected with siLMWPTP for 72 h. The transfections were done using a Hiperfect transfection kit (Qiagen) according to the manufacturer's instructions. The negative control was an irrelevant RNA sequence and the positive control was a sequence directed against MAPK1 (knock down not shown). The cells were subsequently scraped into cell lysis buffer and transfection was confirmed by western blotting. β-Actin was used as a loading control. The bar graph shows the relative amounts of phosphorylated Y<sub>416</sub>Src and LMWPTP expression corrected for β-actin.

#### *LMWPTP dephosphorylates phospho-Src in cells*

To establish whether pSrc can act as a substrate for LMWPTP in intact cells, we examined the level of pY<sub>416</sub> in MC3T3-E1 cells overexpressing LMWPTP or empty vector. As shown in Figure 4, the cells generally had low basal levels of pSrc. To increase the levels of pY<sub>416</sub>Src, the cells were preincubated for 30 min with pervanadate before transfection with empty vector or an LMWPTP-expressing construct and then incubated in medium without pervanadate. Comparison of the upper panel with the middle panel in Figure 4A, or the western blots in Figure 4B shows that this short pretreatment was sufficient to substantially increase pY<sub>416</sub>Src levels in cells transfected with empty vector, even after 24 h. Transfection with an LMWPTP construct (lower panel) resulted in no detection of pY<sub>416</sub>Src. These results clearly show that pSrc is an intracellular substrate for LMWPTP.

#### *LMWPTP is essential for suppressing Src hyperphosphorylation in cells*

The experiments described in the preceding section indicated that LMWPTP reduced Src phosphorylation, but did not address the importance of this action *in vivo*. To examine this aspect, LMWPTP expression was

knocked down by using siRNA. A reduction in the intracellular levels of LMWPTP markedly enhanced Src phosphorylation (Figure 5). Hence, LMWPTP not only reduces Src phosphorylation intracellularly, but also suppresses Src hyperphosphorylation.

#### *The interaction of LMWPTP with Src decreases Src kinase activity*

The apparently antagonistic actions of LMWPTP at both regulatory sites of Src raised questions as to the actual effects of LMWPTP on Src catalytic activity. To investigate this question, Src was dephosphorylated with LMWPTP *in vitro* and kinase assays were done using Sam68 as a substrate for Src to monitor the effect of LMWPTP on Src activity. The level of tyrosine phosphorylation of Sam68 was monitored by immunoblotting with pY<sub>20</sub> anti-phosphotyrosine antibody. As shown in Figure 6, Sam68 was efficiently phosphorylated by Src when this kinase was not pre-incubated with LMWPTP. However, after a 30 min pre-incubation with LMWPTP, the ability of Src to phosphorylate Sam68 was almost completely abolished. These experiments unambiguously identified LMWPTP as a negative regulator of Src enzymatic activity.



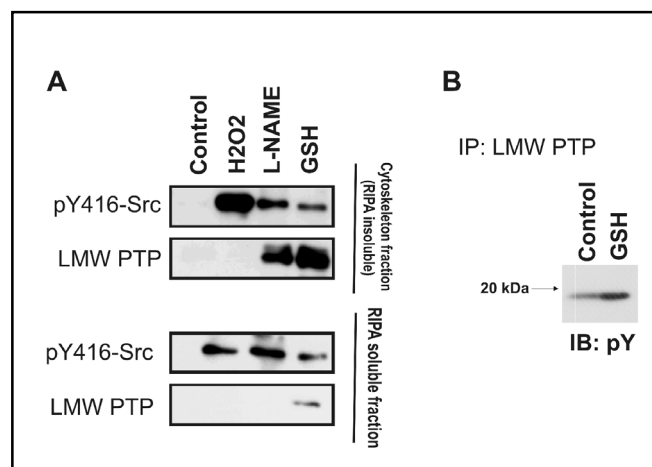
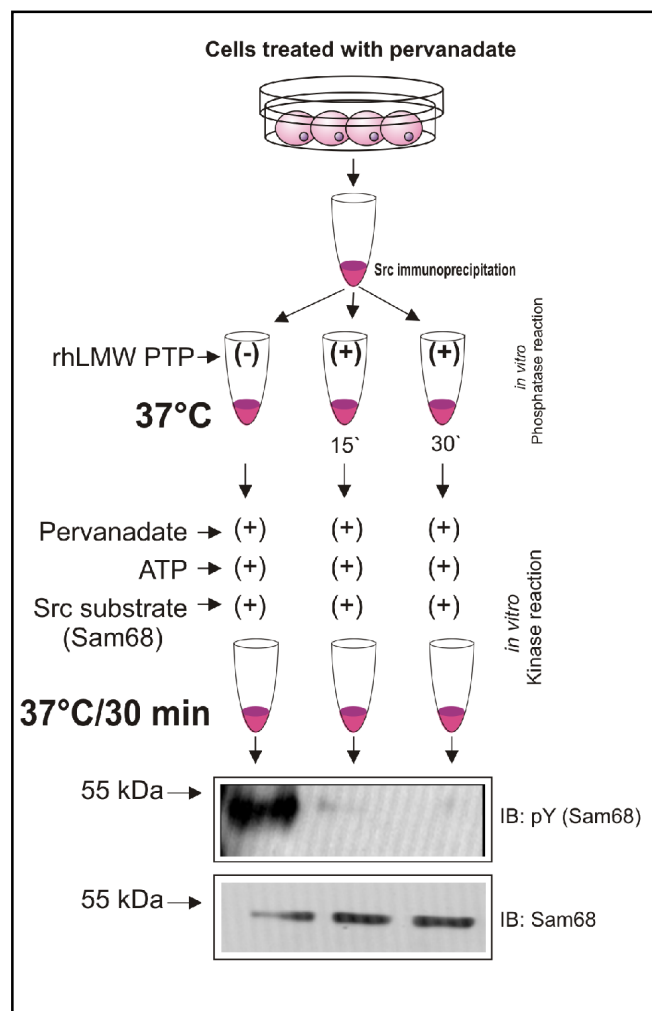
**Fig. 6.** LMWPTP modulates Src kinase activity. Src kinase was immunoprecipitated and incubated with rhLMWPTP for 15 min or 30 min, followed by the addition of 10  $\mu$ M pervanadate, Sam68 (Src substrate) and ATP. After incubation for 30 min, the reaction was terminated and the levels of Sam68 and its phosphorylation were assessed by western blotting.

*Redox status affects the subcellular distribution of LMWPTP and Src*

In view of the importance of the redox status of cells in modulating the intracellular localization and activity of PTP in general, we examined the effect of  $H_2O_2$ , L-NAME and GSH on the intracellular distribution and activity of LMWPTP. Changes in the cellular redox potential resulted in the translocation of LMWPTP to the RIPA-insoluble cytoskeletal fraction (Figure 7A). When the reducing potential of cells was increased by adding L-NAME or GSH, LMWPTP was translocated to the soluble fraction (cRIPA) - (Figure 7A). Under the same conditions, there was a decrease in pYsrc levels that was inversely related to the increase in LMWPTP levels. Hence, LMWPTP was negatively correlated with pYsrc, also on a subcellular level. Cells incubated with GSH showed higher LMWPTP phosphorylation (Figure 7B). Together, these results provide further evidence for a role of this phosphatase in controlling the phosphorylation of c-Src.

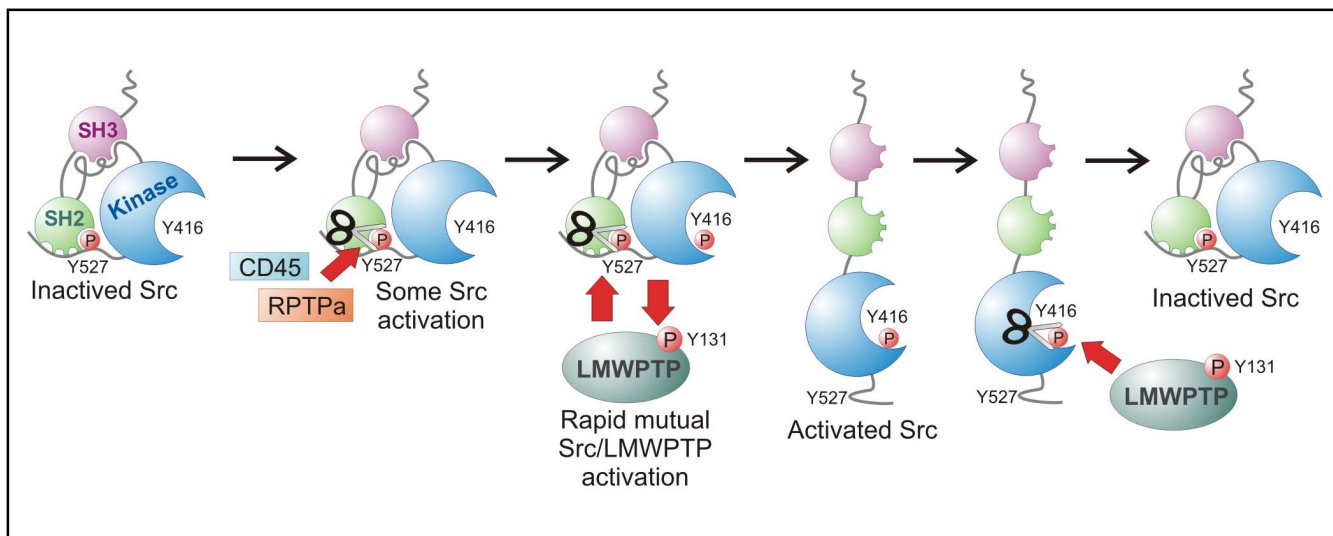
## Discussion

Despite the importance of Src kinase in cellular physiology, including bone homeostasis, many aspects of the regulation of this enzyme by covalent modulation (via Y<sub>416</sub>Src and Y<sub>527</sub>Src) remain unclear. Since previous reports have demonstrated a role for Src in osteoblast differentiation [3-5], in this work we examined the possible correlation between Src phosphorylation and LMWPTP expression during this process in pre-osteoblasts stimulated with ascorbic acid and  $\beta$ -glycerophosphate. Firstly, Src activity was inhibited during differentiation process, as would be expected, whereas LMWPTP expression was markedly increased. This finding suggested a possible relationship between them. Additional support for such a relationship was obtained from experiments in which MC3T3-E1 cells were treated with pervanadate. Although such treatment would be expected to negatively regulate pY<sub>527</sub>Src dephosphorylating enzymes and thereby



**Fig. 7.** LMWPTP activity is modulated by the cellular redox status. (A) MC3T3 cells were treated with hydrogen peroxide, L-NAME or GSH for 30 min and cells were fractionated into RIPA soluble and insoluble fractions. The presence of LMWPTP and pY<sub>416</sub>-Src was evaluated in both fractions. (B) Cells treated with GSH were lysed and LMWPTP was immunoprecipitated, followed by determination of pY-LMWPTP using  $\alpha$ -pY antibody.





**Fig. 8.** Schematic representation of the modulation of Src activity by dephosphorylation. The phosphorylation of Y<sub>527</sub> in Src is crucial for maintaining this enzyme in its inactive conformation since pY<sub>527</sub> interacts with the SH2 domain of the kinase and this intramolecular association stabilises a catalytically inactive form of the enzyme. Following dephosphorylation of Y<sub>527</sub>Src by CD45/RPTP $\alpha$ , Src undergoes intermolecular autophosphorylation at Y<sub>416</sub>, a residue present in the activation loop. The phosphorylation of Y<sub>416</sub> activates kinase activity and leads to the association of Src with substrate molecules, e.g. LMWPTP. LMWPTP dephosphorylates Y<sub>527</sub>Src, leading to an increase in kinase activity, in a process defined as rapid mutual activation. When Y<sub>416</sub>Src is dephosphorylated by LMWPTP, Src returns to its inactive conformation.

downregulate pY<sub>416</sub>-Src levels, instead there was increase in pY<sub>416</sub>-Src levels. The simplest explanation for this observation is that these cells contain PTP activity towards pY<sub>416</sub>-Src, making this phosphatase a plausible negative regulator for c-Src activity. Among the PTPs, it has been demonstrated that the LMWPTP is complexed with and a direct substrate for active Src, and the phosphorylation of LMWPTP increased its enzymatic activity [14, 19]. Hence, this enzyme could be an attractive candidate to act as a negative modulator of Src activity. Support for this hypothesis was obtained from experiments in which the subcellular distribution of LMWPTP was assessed in parallel with the phosphorylation of c-Src. The subcellular localization of many enzymes, such as protein kinase C, Raf and Src, is influenced by extracellular stimuli, and detergent phase partitioning is commonly used to isolate these proteins [19, 21-22]. RIPA buffer is a widely used lysis buffer that contains non-ionic detergents such as Triton X-100 or Nonidet P-40. The soluble fraction obtained with RIPA contains cytosolic and plasma membrane structures. As shown here, increasing the redox potential of the cell resulted in the translocation of LMWPTP to the RIPA-insoluble cytoskeletal fraction. This finding agrees with the earlier study of Caselli et al. [23] who showed that the cellular redox status is a

critical determinant of the subcellular localization of LMWPTP, an effect that is probably related to the phosphorylation of Y<sub>131</sub> in LMWPTP. The appearance of LMWPTP in this fraction correlated directly with a decrease in pSrc levels. Furthermore, artificially increasing the expression of LMWPTP resulted in the downregulation of pSrc. These findings indicate that pSrc is a substrate for LMWPTP *in vivo*.

Our results indicate that LMWPTP apparently has two distinct actions in transiently modulating Src activity. On the one hand, modest activation of LMWPTP dephosphorylates pY<sub>527</sub>-Src. This leads to the phosphorylation of Y<sub>416</sub>-Src and a subsequent dramatic increase in PTK activity that in turn results in more LMWPTP tyrosine phosphorylation and hence more phosphatase activity (Figure 8). The inherent positive feedback in this process may explain the rapid increase in cellular Src activity generally seen after cellular stimulation. On the other hand, LMWPTP also dephosphorylates pY<sub>416</sub>-Src, thus temporally limiting Src activity. This double action makes LMWPTP a prime candidate in the remarkable dynamics of Src activation following cellular stimulation.

However, it is possible that the dephosphorylation of pY<sub>527</sub>-Src is an epiphenomenon with little relevance *in*

*vivo*. This hypothesis is supported by results of this study showing that LMWPTP acts as a negative regulator of Src activity. Such a scheme would entail the activation of Src via the dephosphorylation of pY<sub>527</sub>Src by other PTPs, e.g. CD45 or RPTP $\alpha$ . The increased Src phosphorylation would subsequently produce increased LMWPTP activity via Y<sub>131/132</sub> phosphorylation. This in turn would negatively modulate Src via dephosphorylation of pY<sub>416</sub>Src.

Both of these possibilities are not mutually exclusive and may act in parallel. This is an aspect that merits further investigation. In addition, it would be interesting to establish the extent to which unphosphorylated Y<sub>416</sub>/Y<sub>517</sub>Src can serve as a substrate for Y<sub>527</sub>Src phosphorylation by Csk [24]. Regardless of the precise pathway by which LMWPTP regulates Src activity, the present study has shown that this PTP is a dualistic regulator of Src activity and identifies LMWPTP as the first bonafide negative regulator of Y<sub>416</sub>Src phosphorylation.

Taking in consideration the importance of Src and protein tyrosine phosphatases in the bone metabolism [2-5, reviewed 25], the results described here provide new information on the modulation of Src kinase activity. Src activity, but not expression, was significantly altered during osteoblast differentiation. The changes in Src activity were apparently mediated by the action of LMWPTP on this kinase. The widespread occurrence of both of these enzymes in animal cells suggests that the regulatory mechanisms described here may also occur in other cell types. In addition, further investigation of

the interaction between Src and LMWPTP could provide new approaches for developing novel drugs for bone diseases.

## Abbreviations

CD45 (cluster of differentiation/receptor protein tyrosine phosphatase); cRIPA (cytoskeletal fraction); L-NAME (N<sup>G</sup>-nitro-L-arginine methyl ester); LMWPTP (low molecular weight protein tyrosine phosphatase); PDGF (platelet-derived growth factor); PTK (protein tyrosine kinase); PTP (protein tyrosine phosphatase); pY (phospho-tyrosine); RPTP $\alpha$  (receptor protein tyrosine phosphatase  $\alpha$ ); Sam68 (substrate for Src); Src (Rous sarcoma); Y (tyrosine residue).

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